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Proceedings of the National Academy of Sciences of the United States of America, Vol.
86, No. 21 (Nov. 1, 1989), 8222-8226.

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Functional comparison of the Rev trans-activators encoded by different primate immunodeficiency virus species

(AIDS/post-transcriptional regulation/RNA structure)

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Communicated by W. K. Joklik, July 21, 1989

ABSTRACT The known primate lentiviruses can be divided into two subgroups consisting of the human immunodeficiency virus type 1 (HIV-1) isolates and the related HIV type 2 (HIV-2) and simian immunodeficiency virus (SIV) isolates. HIV-1 has been shown to encode a post-transcriptional trans-activator of viral structural gene expression, termed Rev, that is essential for viral replication in culture. Here, we demonstrate that HIV-2 and SIV_{mac} also encode functional Rev proteins. As in the case of HIV-1, these Rev trans-activators are shown to induce the cytoplasmic expression of the unspliced viral transcripts that encode the viral structural proteins. Unexpectedly, the Rev proteins of HIV-2 and SIV_{mac} proved incapable of activating the cytoplasmic expression of unspliced HIV-1 transcripts, whereas HIV-1 Rev was fully functional in the HIV-2/SIV system. This nonreciprocal complementation may imply a direct role for Rev in mediating the recognition of its viral RNA target sequence.

The pathogenic human retrovirus human immunodeficiency virus (HIV) type 1 (HIV-1) is the major etiologic agent of acquired immunodeficiency syndrome (AIDS) (1). Since the identification of HIV-1, the related lentiviruses human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus (SIV) have been isolated from humans and captive rhesus macaques, respectively (2–6). (The macaque isolate is termed SIV_{mac}.) HIV-2 displays ≈45% nucleotide sequence identity with HIV-1 and ≈75% sequence identity with SIV_{mac} and is therefore believed to be more closely related to the SIVs (2, 6, 7). While HIV-2 shares with HIV-1 the ability to induce cytopathic effects on infected CD4⁺ T lymphocytes in culture (8), HIV-2 appears to be much less pathogenic in human patients than HIV-1 (9). In contrast, SIV_{mac} induces an immune deficiency disease in macaques that is strikingly similar to AIDS, but appears to be asymptomatic in various related Simian species (10, 11). The molecular basis for these differences in pathogenicity remains unknown.

The HIV-1 genome encodes not only the three structural proteins [group-specific antigen (Gag), DNA-dependent RNA polymerase (Pol), and envelope glycoprotein (Env)] common to other known retroviruses, but also at least six other gene products whose functions remain incompletely understood (12). Two of these proteins, termed Tat and Rev, are nuclear trans-activators essential for HIV-1 replication in culture (13–16). It has been shown that both HIV-2 and SIV_{mac} also encode a functional Tat protein that, like HIV-1 Tat, acts to enhance the expression of sequences linked to the viral long terminal repeat (LTR) promoter element (2, 17). In contrast, no studies have as yet demonstrated the existence

of a functional *rev* gene in either HIV-2 or SIV_{mac}. However, open reading frames that are potentially equivalent to the HIV-1 *rev* gene have been defined in both viruses (2, 6).

The HIV-1 Rev trans-activator has been shown to be absolutely required for expression of the viral structural proteins (18, 19). These gene products are encoded by a population of incompletely spliced viral transcripts that are constitutively expressed in the nucleus but excluded from the cytoplasm in the absence of Rev (20–22). In this report, we demonstrate that both HIV-2 and SIV_{mac} encode a functional Rev protein that is able to activate the cytoplasmic expression of unspliced HIV-2 or SIV_{mac} mRNAs. The HIV-1 Rev protein was also found to effectively induce the functional expression of unspliced HIV-2 and SIV_{mac} transcripts. In contrast, the Rev proteins of HIV-2 and SIV_{mac} were observed to be nonfunctional in the HIV-1 system.

MATERIALS AND METHODS

Construction of Molecular Clones. The parental expression vector pBC12/CMV and the HIV-1-based vectors pgTAT(HIV-1) and pgREV1 (previously termed pgTAT and pgREV) have been described (23). A similar strategy was used to construct the genomic HIV-2- and SIV_{mac}-based vectors illustrated in Fig. 1. The published sequence coordinates of restriction sites utilized during these constructions are indicated in parentheses. A proviral clone of HIV-2_{ROD} (2) was used to construct pgTAT(HIV-2) and pgREV2. These contain the *Hind*III(5783)–*Pvu* II(8430) and *Ava* I (6059)–*Bam*HI(8570) fragments, respectively. The SIV_{mac} proviral clone BK28 (6) was used for construction of pgTAT(SIV) and pgREV3. These vectors contain the *Hae* II(602)–*Xmn* I(3160) and *Nla* III(710)–*Bgl* II(3372) fragments, respectively. The pgREV(12) vector was constructed by replacing the *Pvu* II(6662)–*Xho* I(8475) restriction fragment of pgREV1 with a *Tth*III1 (7816)–*Bam*HI(8570) fragment from pgREV2. The related vector pgREV(21) lacks the *Mst* II(5537)–*Bgl* II(7199) fragment of pgREV1 and instead contains an *Ava* I(6059)–*Tth*III1(7752) fragment from pgREV2. The pΔT vector was constructed by deletion of a *Tth*III1 fragment (7752 and 7816) of pgTAT(HIV-2). Similarly, pΔNS was constructed from pgTAT(HIV-2) by the deletion of an *Nci* I(6835) to *Ssp* I(7260) fragment. The human T-cell leukemia virus type I Rex cDNA expression vector pCREX has been described (24).

Cell Culture and Transfection. The HIV-1 replication-competent monkey cell line COS was maintained as described (26). Cells were transfected by using DEAE-dextran and chloroquine (25).

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Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; RRE, Rev response element.

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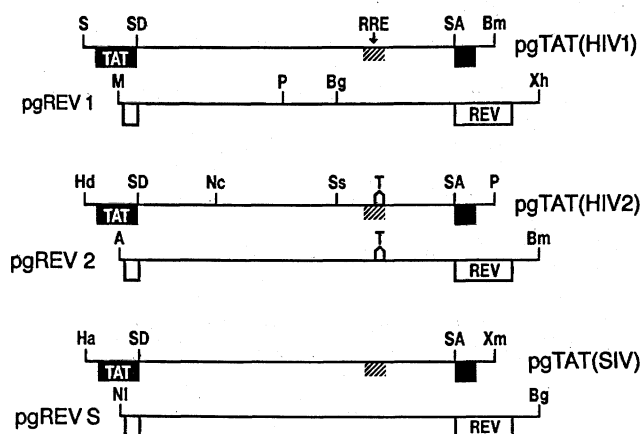


FIG. 1. Structure of the HIV-1, HIV-2, and SIV_{mac} genomic Tat and Rev expression vectors. The indicated full-length proviral restriction fragments were derived from published HIV-1, HIV-2, and SIV_{mac} proviral clones (2, 6, 23) and were inserted into the expression vector pBC12/CMV (23). They are therefore expressed under the control of the Tat-nonresponsive immediate early promoter of cytomegalovirus and flanked 3' by a polyadenylation sequence derived from the genomic rat insulin II gene. The locations of the *tat* exons (solid boxes), *rev* exons (unshaded boxes), Rev response elements (RRE) (hatched boxes), splice donors (SD), splice acceptors (SA), and relevant restriction sites are indicated. S, *Sal* I; Bm, *Bam*HI; M, *Mst* II; P, *Pvu* II; Bg, *Bgl* II; Xh, *Xho* I; Hd, *Hind*III; Nc, *Nci* I; Ss, *Ssp* I; T, *Tth*III; A, *Ava* I; Ha, *Hae* II; Xm, *Xmn* I; NI, *Nla* III.

RNA Isolation and S1 Nuclease Protection Assays. Cytoplasmic RNA was isolated from COS cells 60 hr after transfection and subjected to S1 nuclease protection analysis as previously described (20, 23, 26). DNA probes were end-labeled within the first coding exon of the *tat* gene and extend into the flanking intron. A pBR322-derived tag permitted the distinction of the input probe (I) from the unspliced (U) and spliced (S) RNA signals. The 798-nucleotide HIV-1 *tat* gene probe (Fig. 2A) (23) was labeled at an *Xho* II site located within the first exon of *tat* and extends beyond the *tat* gene splice donor at 202 nucleotides (S) to an intronic *Kpn* I site at 506 nucleotides (U). The 1162-nucleotide SIV_{mac} *tat* gene probe (Fig. 2B) was labeled at an introduced *Hind*III site. It extends through the *tat* gene splice donor at 243 nucleotides (S) to an intronic *Pvu* II site at 270 nucleotides (U).

Antisera and Immunoprecipitation Analysis. We have described a rabbit polyclonal antiserum directed against amino acid residues 1–61 of the HIV-1 Tat protein (26). A similar approach was used to derive a rabbit polyclonal antiserum directed against a synthetic peptide comprising amino acids 75–99 of the HIV-2 Tat protein. Immunoprecipitation of [³⁵S]cysteine-labeled Tat proteins was performed as described (20, 25).

RESULTS

We have previously described the use of a genomic HIV-1 *tat* gene expression vector, pgTAT(HIV-1), as an indicator of Rev function (20, 23) (Fig. 1). In the absence of Rev, pgTAT(HIV-1) predominantly expresses a fully spliced cytoplasmic *tat* mRNA transcript (Fig. 2A, lane 1) that encodes the 86-amino acid two-exon form of the Tat protein (Fig. 3A, lane 1). Cotransfection of cells with the HIV-1 genomic *rev* gene expression vector, pgREV1 (Fig. 1), induces the cytoplasmic expression of an unspliced *tat* mRNA (Fig. 2A, lane 2), structurally equivalent to *env* mRNA, that encodes a truncated, one-exon form of the Tat protein comprising 72 amino acids (Fig. 3A, lane 2).

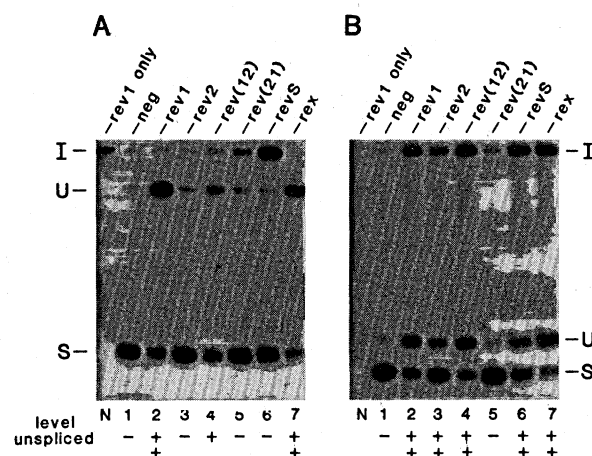


FIG. 2. S1 nuclease protection analysis of cytoplasmic *tat* mRNA. Assays were performed with 5 μ g of transfected cell RNA and utilized DNA probes that distinguished the full-length input probe (I) from the fragments rescued by the unspliced (U) and spliced (S) *tat* gene transcripts (20, 23). The relative responsiveness of the genomic HIV-1 *tat* gene expression vector pgTAT(HIV-1) (A) and the equivalent SIV_{mac} *tat* gene expression vector pgTAT(SIV) (B) to the coexpressed Rev trans-activators is summarized below each lane: ++, full response (>40% unspliced); +, partial response (10–40% unspliced); –, no response (<10% unspliced). The culture used for each lane N was transfected with pgREV1 alone and served as a control for probe specificity, while pBC12/CMV served as a negative (neg) control.

To determine whether functional Rev proteins are encoded by HIV-2 and SIV_{mac}, we constructed analogous genomic *tat* and *rev* expression vectors using HIV-2 and SIV_{mac} proviral sequences (2, 6) (Fig. 1). We predicted that, as with pgTAT(HIV-1), Rev responsiveness of pgTAT(HIV-2) and pgTAT(SIV) would be reflected by the induced cytoplasmic expression of unspliced HIV-2 and SIV_{mac} *tat* gene transcripts. We therefore performed S1 nuclease protection assays on cytoplasmic RNA isolated from COS cell cultures transfected with pgTAT(SIV) in the presence or absence of the various *rev* gene expression vectors. As anticipated, pgTAT(SIV) was observed to predominantly express a fully spliced cytoplasmic SIV_{mac} *tat* gene transcript in the absence of Rev (Fig. 2B, lane 1). In contrast, cotransfection of any of the Rev expression vectors induced the expression of a significant level of unspliced cytoplasmic SIV_{mac} *tat* mRNA (Fig. 2B, lanes 2, 3, and 6). Therefore, HIV-2 and SIV_{mac} do indeed encode a functional Rev protein that is able to activate the cytoplasmic expression of unspliced SIV_{mac} transcripts.

A translation stop codon located immediately downstream of the first exon of the *tat* gene is conserved among all known primate immunodeficiency viruses (20). After the Rev-induced cytoplasmic expression of unspliced HIV-1 *tat* transcript, this stop codon is utilized to yield a truncated, 72-amino acid HIV-1 Tat protein (Fig. 3A, lane 2). We therefore asked whether Rev could also induce the synthesis of a truncated HIV-2 Tat protein. The full-length, two-exon form of the HIV-2 Tat protein comprises 130 amino acids, while the single-exon form is predicted to consist of 99 amino acids (2). To generate the antiserum specific for these HIV-2 Tat proteins, rabbits were immunized with a synthetic peptide comprising amino acids 75–99 of HIV-2 Tat. The specificity of this rabbit anti-Tat(HIV2) antiserum was established by its ability to immunoprecipitate a \approx 21-kDa protein from metabolically labeled COS cells transfected with the HIV-2 Tat expression vector, pgTAT(HIV-2) (Fig. 3B, lane 1) that was absent from a control culture (Fig. 3B, lane N). Although this apparent molecular mass is surprisingly high for a protein of 130 amino acids, we note that the 86-amino acid HIV-1 Tat

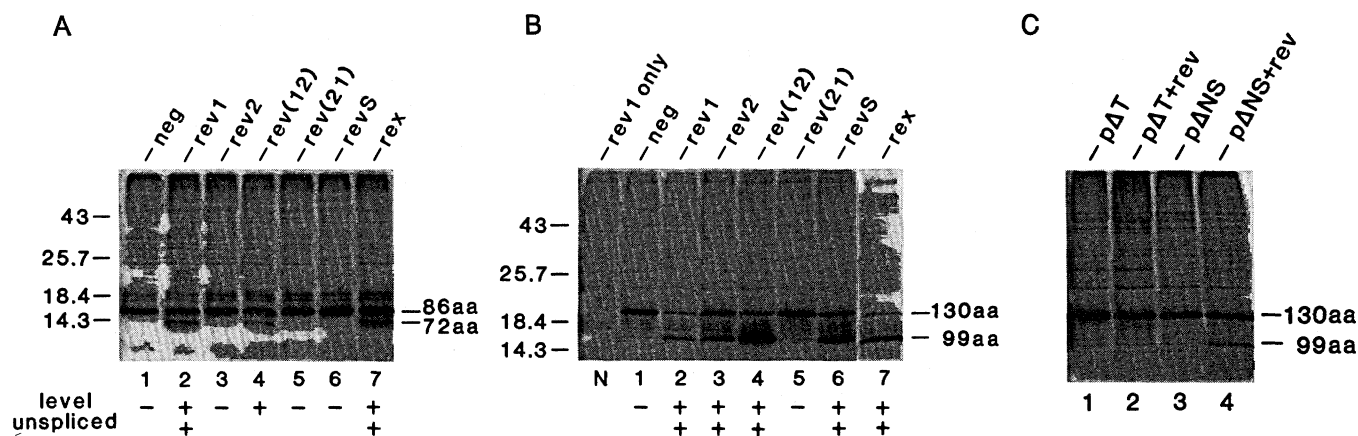


Fig. 3. Immunoprecipitation analysis of HIV-1 and HIV-2 Tat proteins. Labeled proteins derived from the transfected COS cell cultures were immunoprecipitated by using anti-Tat(HIV-1) (A) or anti-Tat(HIV-2) antiserum (B and C). Precipitated proteins were resolved on discontinuous SDS/14% polyacrylamide gels and visualized by autoradiography (23). The predicted size of each species of Tat protein is indicated. Numbers on the left are positions of marker proteins in kDa. The responsiveness of the unspliced HIV-1 and HIV-2 *tat* gene transcripts to the coexpressed viral Rev trans-activators is reflected by the induced synthesis of the truncated 72-amino acid (72 aa) (HIV-1) or 99aa (HIV-2) forms of Tat (20, 23). For A and B, the extent of each response is summarized below each lane: ++, full response (>20% one-exon Tat); +, partial response (5–20% one-exon Tat); –, no response (<5% one-exon Tat). The parental vector pBC12/CMV served as a negative control (neg). (A) Cultures were transfected with pgTAT(HIV-1) and the indicated test plasmid. (B) All cultures, except N, were transfected with pgTAT(HIV-2) and the indicated test plasmid. (C) Cultures were transfected with the indicated vectors.

protein also migrates unusually slowly in SDS/polyacrylamide gels (Fig. 3A, lane 1). As predicted, coexpression of any of the viral Rev trans-activators resulted in the expression of a high level of a truncated ≈ 17 kDa form of the HIV-2 Tat protein (Fig. 3B, lanes 2, 3, and 6) that was essentially absent from the culture transfected with the pgTAT(HIV-2) vector alone (Fig. 3B, lane 1). Thus, as is the case with HIV-1, the synthesis of a foreshortened HIV-2 Tat protein correlates with the induced cytoplasmic expression of an unspliced HIV-2 *tat* gene transcript.

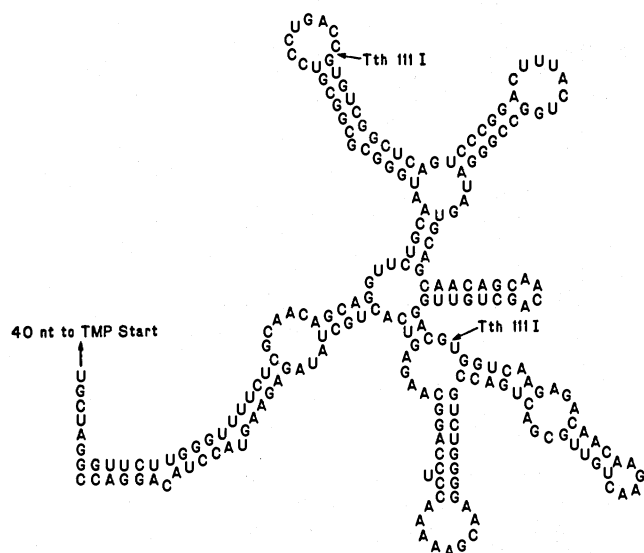
We next asked whether the Rev proteins encoded by HIV-2 or SIV_{mac} would also induce the cytoplasmic expression of unspliced HIV-1 *tat* gene transcripts. To address this possibility, COS cell cultures were transfected with pgTAT(HIV-1) in the presence of either pgREV2 or pgREV5. Potential Rev responsiveness was then assessed by S1 nuclease protection analysis of cytoplasmic RNA (Fig. 2A) and by the immunoprecipitation of labeled HIV-1 Tat protein with anti-Tat(HIV-1) antiserum (Fig. 3A). Remarkably, this experiment showed that the Rev proteins of HIV-2 and SIV_{mac} were unable to activate the cytoplasmic expression of unspliced HIV-1 *tat* transcript (Fig. 2A, lanes 3 and 6) and therefore did not induce the detectable synthesis of the 72-amino acid HIV-1 Tat protein that this mRNA encodes (Fig. 3A, lanes 3 and 6). Thus it appears that, whereas HIV-1 Rev can induce the expression of unspliced viral transcripts derived not only from HIV-1 but also from HIV-2 and SIV_{mac}, the Rev proteins of HIV-2 and SIV_{mac} are able to functionally interact only with unspliced viral transcripts derived from the related HIV-2 and SIV_{mac} proviruses.

To map the region of the HIV-2 Rev protein responsible for its failure to activate the cytoplasmic expression of the unspliced HIV-1 *tat* gene transcript (Fig. 2A, lane 3), two hybrid genomic Rev expression vectors, pgREV(12) and pgREV(21), were constructed by exchanging the first and second exons of pgREV1 and pgREV2. pgREV(12) therefore contains the first exon of HIV-1 *rev* and the second exon of HIV-2 *rev*, while pgREV(21) comprises the first exon of HIV-2 *rev* and the second exon of HIV-1 *rev*. The unspliced HIV-1 *tat* gene transcript was found to be partially responsive to the hybrid Rev(12) protein, as visualized by its enhanced expression in the cell cytoplasm (Fig. 2A, lane 4) and by the induced synthesis of 72-amino acid HIV-1 Tat (Fig. 3A, lane 4). It should be noted that the first exon of the

HIV-1 *rev* gene is not sufficient for Rev response, as mutations in the second exon of the HIV-1 *rev* gene are known to abolish Rev function (20–22, 27). Earlier we demonstrated that HIV-2 and SIV_{mac} were responsive to the Rev proteins of both HIV-1 and HIV-2 (Figs. 2B and 3B, lanes 2 and 3). The observation that the cytoplasmic expression of these two transcripts was induced by the Rev(12) hybrid protein was therefore anticipated (Figs. 2B and 3B, lanes 4). It was, however, surprising that the Rev(21) protein did not activate the cytoplasmic expression of unspliced HIV-2 or SIV_{mac} transcripts (Figs. 2B and 3B, lanes 5). However, we have recently found that the chimeric Rev protein encoded by pgREV(21) is highly unstable (data not shown). Consequently this nonresponsiveness is at present uninterpretable.

The human T-cell leukemia virus type I (HTLV-I) encodes a viral trans-activator, termed Rex, that can functionally replace the HIV-1 Rev protein (24). We therefore asked whether Rex might also activate the cytoplasmic expression of unspliced *tat* transcripts derived from HIV-2 or SIV_{mac}. As previously shown, the unspliced HIV-1 *tat* transcript was fully responsive to Rex (Figs. 2A and 3A, lanes 7). Interestingly, unspliced *tat* transcripts derived from both HIV-2 and SIV_{mac} were also observed to be fully responsive to the HTLV-I Rex protein (Figs. 2B and 3B, lanes 7). Hence it appears that Rex is able to functionally substitute for the Rev proteins of all the primate immunodeficiency viruses tested here.

The target sequence for Rev in HIV-1, termed the Rev response element or RRE, precisely coincides with a region of the viral *env* gene that is predicted to form a highly stable RNA secondary structure (20). The Rev responsiveness of pgTAT(HIV-2) and pgTAT(SIV) prompted us to ask whether the HIV-2 and SIV_{mac} *env* genes might also contain regions capable of forming significant RNA secondary structures. A detailed computer analysis of these regions was therefore performed, and the most stable RNA secondary structure predicted for the HIV-2 *env* gene is presented in Fig. 4. A virtually identical structure was also recognized in the SIV_{mac} *env* gene (data not shown). This HIV-2-derived sequence consists of 216 nucleotides and, like the HIV-1 structure, is located within the region of *env* that encodes the N terminus of the transmembrane glycoprotein (20). The striking similarity in the complexity, overall appearance, and location of these two RNA structures suggested that a high degree of



We have shown that the genomic HIV-2 Tat expression vector pgTAT(HIV-2) is responsive to Rev and that HIV-2 *env* gene sequences present in pgTAT(HIV-2) contain a sequence predicted to form a highly stable RNA secondary structure. In HIV-1, the RRE coincides with this predicted

DISCUSSION

In light of the extensive similarity in the function of the Rev proteins of HIV-1, HIV-2, and SIV_{mac} delineated above, it was surprising when our further analysis revealed the incomplete ability of these viral Rev proteins to cross-complement the expression of unspliced viral mRNA. In particular, all the viral Rev trans-activators were able to function within the HIV-2 and SIV_{mac} contexts while only the HIV-1 Rev protein could induce the cytoplasmic expression of unspliced HIV-1 mRNAs (Figs. 2A and 3A). This nonreciprocity suggests that the activation function of Rev is encoded by a Rev

HIV-1	1	AGGAGCUUU---C---C---G-G-G---AAGCA-U-----A-----AA---GC-A---UA---G---	
HIV-2	1GGU- CUUGGGUUUUUCGCAACAGCAGGUUCUGCAAUGGGCGCGGCUCUGACCGUGUGGGCUCAGUCC	
SIV	1	-----A-----G-----A-----GU---GC---A-C-----	
HIV-1	80	A-ACAA---U-U-U---G---A-CA-UU---C---AGG-CUA-UG---GCG---GC-U---A-----	
HIV-2	72	CGGACUUUACUGGCGCGGAUAGUGCAGCAACAGCAACAGCUUGGACGUGGUAAGAGACAACAGAAUCUGUUGCGACU	
SIV	72	-----U-----U-----U-----	
HIV-1	161	C---A-----C-UC---GC-G-----A---CUG---G-G---A-GA-----A---U-AACAGCUCCU	234
HIV-2	153	GACCGUCUGGGGAACGAAAAACUCCAGGCAAGAGUACUGCUAAUAGAGAAUACCUACAGAGACC.....	216
SIV	153	-----A-G-----A-U-A-G-----C-C-----	216

FIG. 5. Alignment of the sequences that comprise the predicted RNA secondary structures within the *env* genes of HIV-1, HIV-2, and SIV_{mac}. The HIV-1 RRE structure is 234 nucleotides in length, while the structures of HIV-2 and SIV_{mac} are both predicted to be 216 nucleotides long. The complete RNA sequence of the HIV-2 structure is shown, and nucleotide differences present in the HIV-1 and SIV_{mac} sequences are marked (2, 6, 20). Conserved nucleotides are represented with a dash (-), while dots (·) signify the absence of corresponding nucleotides at the 5' and 3' ends of the HIV-2 and SIV_{mac} sequences. This is due to the smaller size of these predicted RNA structures. In addition, dots mark the locations of the two single-nucleotide shifts that were included to obtain the maximum number of nucleotide identities.

protein sequence at least partly distinct from the amino acid sequence that confers sequence specificity. In an initial attempt to map this latter domain, we constructed vectors predicted to encode chimeric HIV-1/HIV-2 Rev proteins. A Rev protein derived from the first exon of HIV-1 and the second exon of HIV-2, termed Rev(12), proved functional in both the HIV-1 and HIV-2 systems. One interpretation of this result is that the 25-amino acid first exon of HIV-1 confers at least a part of the sequence specificity inherent in the HIV-1 Rev response. Clearly, however, a more definitive demonstration of the validity of this interpretation will require the construction of an extensive series of Rev mutants and chimeras. Nevertheless, the nonreciprocal complementation noted for the Rev proteins examined here does appear to support the recent hypothesis (27) that the recognition of a particular viral RRE may be a direct, rather than an indirect, function of the Rev trans-activator itself.

We thank Michael Emerman for the gift of the HIV-2_{ROD} proviral clone and James Mullins for the gift of the SIV_{mac} clone BK28. We also thank Mildred McAdams and Richard Randall for synthesis of the HIV-2 Tat peptide and Sharon Goodwin for secretarial assistance.

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